THE EFFECTS OF 5 DAYS OF BED REST ON INSULIN SENSITIVITY AND CERAMIDE BIOSYNTHESIS EXPRESSION IN SKELTAL MUSCLE OF OLDER ADULTS

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Physical inactivity in older adults is a risk factor for developing glucose intolerance and impaired skeletal muscle function. Elevated ceramide levels have been linked to metabolic disruption and toll-like receptor 4 (TLR4) signaling. The purpose of this study is to determine if short-term physical inactivity, bed rest, affects insulin sensitivity and skeletal muscle levels of ceramide biosynthesis gene expression in older adults. We hypothesize that physical inactivity will increase skeletal ceramide biosynthesis expression and decrease Akt phosphorylation in older adults. Secondly, 5 days of bed rest will decrease insulin sensitivity and glucose tolerance and increase NEFA circulation levels in older adults.

Therefore, we recruited 9 healthy male and female older adult participants (age range: 60-75). Participants underwent a 5-day bed rest experiment in the Center for Clinical and Translational Science at the University of Utah Hospital. Muscle biopsies and fasting blood samples were collected. OGTTs were performed.

We found that 5 days of bed rest in older adults resulted in whole body glucose dysregulation, impaired skeletal muscle insulin signaling, and upregulation of markers of ceramide biosynthesis ($P<0.05$). Post bed rest TLR4 abundance was tightly correlated with impaired post-prandial insulin and glucose levels.

In conclusion, 5 days of bed rest in older adults can increase SPT2 protein expression and impair skeletal muscle insulin signaling via Akt phosphorylation. TLR4-mediated SPT2 expression after bed rest may be an important link between physical inactivity and glucose intolerance.

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INTRODUCTION

Physical inactivity is among the leading causes of cardiovascular disease, type 2 diabetes, and certain types of cancer, and contributes considerably to the world-wide spread of disease, death, and disability (1). Intermittent periods of disuse, such as bed rest, or periods of reduced ambulatory activity may have a detrimental effect on metabolic health in older adults (2). One of the early indicators of metabolic disturbances following short-term physical inactivity/bed rest is systemic insulin resistance (2),(3).

Insulin resistance is a factor largely responsible for many metabolic diseases such as type 2 diabetes mellitus, hypertension, atherosclerosis, and cancer (4). Because of the major contribution skeletal muscle has on glucose disposal via insulin action, skeletal muscle can have significant influence on whole body insulin sensitivity. However, the cellular mechanisms are unclear that link altered muscle insulin sensitivity with decreased physical activity in older adults.

Toll-like receptor 4 (TLR4) is a proinflammatory receptor, which is activated by several ligands such as free fatty acids, and has been proposed to mediate insulin resistance by the induction of ceramide biosynthesis (5) (Fig. 1). Therefore, the purpose of this study is to determine if short-term physical inactivity in the form of controlled bed rest affects insulin sensitivity and skeletal muscle levels of ceramide biosynthesis gene expression in older adults.

Insulin resistance in inactive older adults

Older adults are far more likely to be hospitalized, engage in sedentary behavior, or experience prolonged recovery due to injury than younger adults. Physical inactivity, due to these events, can lead to insulin resistance (2), (3), (6). A cross-sectional study by Amati et al. showed that insulin sensitivity was not associated with age regardless of whether young and older adults were athletes, normal-weight subjects, or obese subjects (7). However, a key

observation was that insulin sensitivity was greatest among younger and older athletes and worse in younger and older obese subjects.

A recent study by Drummond and colleagues evaluated insulin impairment (fasting serum levels of glucose and insulin) and leg muscle volume (MRI) in active, healthy and inactive, frail older women (activity levels were assessed by the Physical Activity Scale for the Elderly) (8). Importantly, neither group had any reported underlying medical conditions, including type 2 diabetes (8). In the study, researchers observed lower lean mass and higher fasting insulin and glucose levels in the inactive versus the active older adults, respectively (8). Therefore, insulin resistance is not associated with age but rather the level of physical activity and worsens with obesity (7). Further, insulin resistance can occur independent of any underlying medical condition.

Development of insulin resistance following short-term physical inactivity

Insulin resistance develops when a normal dose of insulin is unable to stimulate the uptake and storage of glucose within cells while at the same time repressing glucose from leaving the liver. However, because of the large mass of skeletal muscle, this organ has major influence on whole body glucose homeostasis. The importance of lean tissue on insulin sensitivity is supported by previous bed rest studies in young healthy adults (9), (10), (11). Stuart and workers found that 7 days of bed rest worsened glucose tolerance and increased endogenous insulin secretion by 40% in response to an oral glucose tolerance test (OGTT) in young participants (9). Nevertheless, physical inactivity did not alter hepatic glucose output nor was there any measureable change in insulin receptor abundances on immune cells (9). Together, these data suggest that short-term physical inactivity-induced, insulin resistance is likely driven by glucose impairments in skeletal muscle.

Insulin resistance is not an event that only occurs over decades of sedentary behavior. Rather insulin resistance can occur within days of being inactive. The time course of insulin resistance during short-term physical inactivity is unclear but appears to be around 3 days of bed rest (measured at day 3 of a 20-day bed rest study) in young healthy subjects (12) while 2 days of

bed rest (13) does not alter insulin sensitivity. In another 3-day bed rest study involving young participants, none of the subjects exhibited impaired glucose tolerance during an OGTT, although there was an increase in plasma insulin in response to glucose ingestion (14). It is unknown if older adults develop insulin resistance in less than a week of bed rest like their younger counterparts (9). This is important because a majority of older adults hospitalized for acute illness typically spend 5-6 days inactive during hospitalization (15).

However, it is known that short-term physical inactivity in the form of bed rest of at least 10 days can significantly impair insulin sensitivity in older adults. Coker et al. found that peripheral and hepatic insulin sensitivity deteriorated significantly following 10 days of controlled bed rest in older, overweight individuals (3). These findings were complemented with an elevation in fasting plasma glucose as well as insulin-mediated suppression of glucose production (3). In another study, Breen et al. demonstrated that 14 days of reduced physical activity (from 5962 to 1413 daily steps) in healthy, normal weight, older men and women induced a significant reduction in insulin sensitivity (as measured by an OGTT) and increased fasting levels of insulin (2). Additionally, insulin resistance preceded overt body composition changes and the onset of metabolic disease (2), (16). Taken together, these data suggest that insulin resistance can develop within 10 days following a period of controlled bed rest in older adults without any reported medical condition. Even a reduction in daily ambulation can lead to rapid impairments in skeletal muscle insulin-mediated sensitivity in healthy older adults. However, it is unknown if less than 10 days of bed rest can induce insulin resistance in healthy older adults. Further it is unknown if the magnitude of insulin resistance to short-term physical inactivity is different between young and older adults matched by BMI and prior activity level.

Cell signaling and insulin resistance: Role of Akt in muscle glucose uptake

Although the literature supports that short periods of physical inactivity can induce insulin resistance in older adults, the cellular events that are responsible for dysregulated glucose uptake following short-term physical inactivity in older skeletal muscle are not well described. From cross-sectional studies, peripheral insulin resistance is characterized by impaired glucose

transport (GLUT4 translocation) in skeletal muscle in the presence of hyperglycemic conditions (hyperinsulinemic clamp procedure) in adults with type 2 diabetes (17). Further, impaired GLUT4 translocation is seen in skeletal muscle of type 2 diabetic patients with peripheral insulin resistance, when the hyperinsulinemic clamp technique was used, and the perpetual hyperglycemia seen in these patients results in resistance to any further insulin-induced plasma membrane-level gain of GLUT4 (17).

In order to understand mechanisms that are upstream of GLUT4 translocation that may be impaired following short-term physical inactivity, a basic understanding of insulin signaling is necessary. Insulin binds to the insulin receptor inducing autophosphorylation of the receptor. The activated receptor phosphorylates a family of IRS proteins that provide binding sites for the p85 subunit of 3-phosphoinositide kinase (18). Akt/protein kinase B (PKB) and phosphatidylinositol-3-phosphate dependent kinase1 are serine/threonine kinases that are brought close to each other by their interactions with PIP3. The membrane lipid helps to activate Akt/PKB, by making conformational changes that uncovers two regulatory phosphorylation sites, Thr308 and Ser473 (19). For full activation of Akt/PKB, there must be phosphorylation of Thr308 (in the activation loop) and also phosphorylation of Ser473 (in the hydrophobic motif) (20). In order for insulin-stimulated translocation of the glucose transporter GLUT4 to the plasma membrane, Akt/PKB must phosphorylate AS160 (21).

Skeletal muscle insulin-resistant individuals and type 2 diabetic patients have impaired insulin-stimulated Akt phosphorylation (Thr308 and Ser473), lower insulin-stimulated AS160 phosphorylation on multiple sites thus leading to impaired GLUT4 translocation (22). Glycogen synthase phosphorylation downstream of Akt is maintained even though there is lower Akt phosphorylation (22). This is evidenced by the impaired glycogen activity in type 2 diabetic patients shown as glycogen synthase site 2+2a (in the NH2-terminus) that becomes hyperphosphorylated during insulin stimulation whereas in glycogen synthase site 3a (in the COOH-terminus), phosphorylation is unchanged (22).

In terms of short-term periods of physical inactivity that induce insulin resistance, dysregulated skeletal muscle Akt signaling can be developed in young subjects after 7 days of

bed rest (6). In this study, bed rest-induced insulin resistance was associated with reduced insulin-stimulated muscular glycogen synthase activity and Akt signaling as well as decreased GLUT4 protein levels (6). Taken together, these data suggest that GLUT4 translocation is diminished in healthy young skeletal muscle following short-term inactivity whereas molecular events associated with insulin resistance are partly related to Akt signaling. It is unknown if Akt signaling is disrupted in skeletal muscle following short-term inactivity (bed rest) in healthy older adults.

Cell signaling and insulin resistance: Role of TLR4 signaling and ceramide biosynthesis

Recent evidence has suggested an alternate mechanism that may be related to resistance and deficient Akt signaling in skeletal muscle. The Toll-like receptor 4 (TLR4) protein is a member of the TLR family and has been generally understood as a proinflammatory signaling pathway. Ligands such as saturated fatty acids are recognized by TLR4 to induce a signaling cascade leading to the activation of nuclear factor κB (NF-κB) and the production of proinflammatory cytokines (5).

TLR4 is located on the cell surface and the activation of the TLR signaling pathway originates from the cytoplasmic Toll/IL-1 receptor (TIR) domain that associates with myeloid differentiation primary response 88 (MyD88). Upon TLR4 stimulation by ligands, MyD88 recruits IL-1 receptor-associated kinase-4 to TLR4. Interleukin-1 receptor-associated kinase 1 is activated by phosphorylation and associates with TNF receptor associated factor-6, thus activating the IκB kinase (IKK) complex and leading to nuclear localization of active NF-κB and subsequently upregulates transcription of several pro-inflammatory cytokines (e.g., IL-6, tumor necrosis factor-alpha (TNFα), IL-β) (23), (24). Evidence of activation of this signaling pathway was noted following a 7-day bed rest study in healthy older adults in which skeletal muscle TLR4 protein content and NFkB1 and IL6 gene expression were increased (25). Their data suggest that a proinflammatory response exists during short-term physical inactivity in older adults and this may be related to the presence of specific TLR4 ligands (25).

Most individuals with type 2 diabetes have elevated plasma non-esterified fatty acid (NEFA) levels, which are associated with skeletal muscle insulin resistance (26). Even following short-term bed rest indications of heighted levels of fatty acids can be observed. Blanc et al. saw a decrease in fasting plasma NEFAs in young adults after 7 days of bed rest (11). Coker and colleagues observed, during a 10-day bed rest study with older overweight adults and by performing a multistage insulin infusion, a non-significant decrease in the insulin-mediated suppression of free fatty acid-rate-of-appearance after bed rest and, as a result, a small change in plasma free fatty acid (FFA) from pre- to post-bed rest (3). Interesting, Hussey et al. demonstrated that an increase in TLR4 expression and flux through the NF-κB pathway, occurred following a continuous, mild elevation of plasma NEFA in normal-glucose-tolerant individuals (27). Therefore, insulin sensitivity is linked to alterations in the regulation of FFAs and is capable of triggering the TLR4/ NF-κB pathway. However, it is not known if bed rest less than a week in older adults results in elevations in circulating levels of NEFAs and if this response is different from young adults following the same bed rest time course.

The sphingolipid ceramide is a supposed intermediate linking saturated fatty acids to the induction of insulin resistance (28). Holland et al. evaluated the effects of saturated vs. unsaturated fats on insulin-stimulated glucose disposal in the muscle of rats. They determined that TLR4 precedes sphingolipids (i.e., ceramide) production and is necessary for de novo ceramide synthesis in skeletal muscle (5). These authors revealed that the IKKβ/NF-kB signaling is essential for TLR4-mediated insulin resistance and upregulation of biosynthetic genes (e.g., CerS, SPT2) involved in ceramide synthesis (5). Further support comes from preliminary data from Dr. Drummond's laboratory. Dr. Drummond and colleagues investigated the role of TLR4 and ceramide as a mediator of insulin resistance caused by physical inactivity. They showed that 2 weeks of hind limb unloading in mice was sufficient to increase skeletal muscle TLR4/NFκB signaling (TLR4, IκBα), ceramide biosynthesis (SPT2), and induce insulin resistance in WT hind limb unloaded mice compared to ambulatory control mice.

Two mechanisms have been proposed as to how ceramide blocks Akt signaling. The first mechanism consists of ceramide activating protein kinase Cζ (PKCζ), which results in the

phosphorylation of Akt at Thr34 (29). This prevents Akt translocation and recruitment onto plasma membranes. The second mechanism involves ceramide activating Protein phosphatase 2 (PP2A), which dephosphorylates the Akt kinase (29). Blocking ceramide accumulation restores insulin-stimulated Akt phosphorylation, even in the presence of excess saturated fatty acids (i.e., palmitate) (30). Therefore, ceramide through the TLR4 pathway may be a novel mechanism of physical inactivity-induced insulin resistance but it has yet to be determined if genes associated with ceramide biosynthesis are upregulated in skeletal muscle of older adults following a short-term period of bed rest.

Significance of the Problem

Skeletal muscle is a vital organ when considering the maintenance of metabolic health and functional independence in older adults. The level of contractile activity of skeletal muscle plays an important role in the sensitivity of insulin. Periods of reduced physical activity, which occur with greater frequency in older adults due to illness or hospitalization, may have a detrimental effect on metabolic health. When muscle fibers are simultaneously exposed to exogenous fatty acids or lipoprotein-bound triglycerides they become insulin resistant, a metabolic state which predisposes individuals to metabolic disorders such as type 2 diabetes. Saturated fatty acids (SFAs), induce the formation of sphingolipids, like ceramide, which is potent antagonists of insulin action.

Purpose of Research

This purpose of this study is twofold: 1) To determine if 5 days of physical inactivity (bed rest) effects ceramide biosynthesis expression and Akt signaling in older adults skeletal muscle, 2) To determine if 5 days of bed rest effects insulin sensitivity and glucose tolerance (as measured with OGTT, HOMA-IR and Matsuda Index), and NEFA blood circulation levels in skeletal muscle of older adults.

Hypothesis

Five days of bed rest will increase skeletal ceramide biosynthesis expression and decrease Akt phosphorylation in older adults. Secondly, 5 days of bed rest will decrease insulin sensitivity and glucose tolerance and increase NEFA circulation levels in older adults.

Strengths and limitations

This is a well-designed study with strong methods. The blood samples, used to measure fasting levels of insulin, glucose, and fatty acid concentrations, as well as muscle biopsies were taken while fasted. This ensured that the subjects did not have a feeding induced cell signaling response. This study mimics the hospital setting and activity was controlled for.

The main weakness of this study, as with most human studies, involves the ability to recruit subjects. Since a muscle biopsy is not a painless procedure, many subjects did want to participate. This was a minor concern since we have been successful recruiting participants in previous studies. Also being immobilized throughout the 5 days of bed rest was not desirable for the subjects. Two issues with the design of the study could be called into question. The OGTT was administered on day 4 instead of day 5 of bed rest and the hyperinsulinemic euglycemic clamp was not used to measuring insulin stimulation.

Clinical applications

The majority of older adults hospitalized for acute illness typically spend 5-6 days inactive during hospitalization (15). Thus, by examining the effects of bed rest on insulin sensitivity and the gene expression of ceramide biosynthesis, researchers can begin to develop pharmacological interventions to target this pathway and prevent the physical inactivity-induced insulin resistance in older adults.

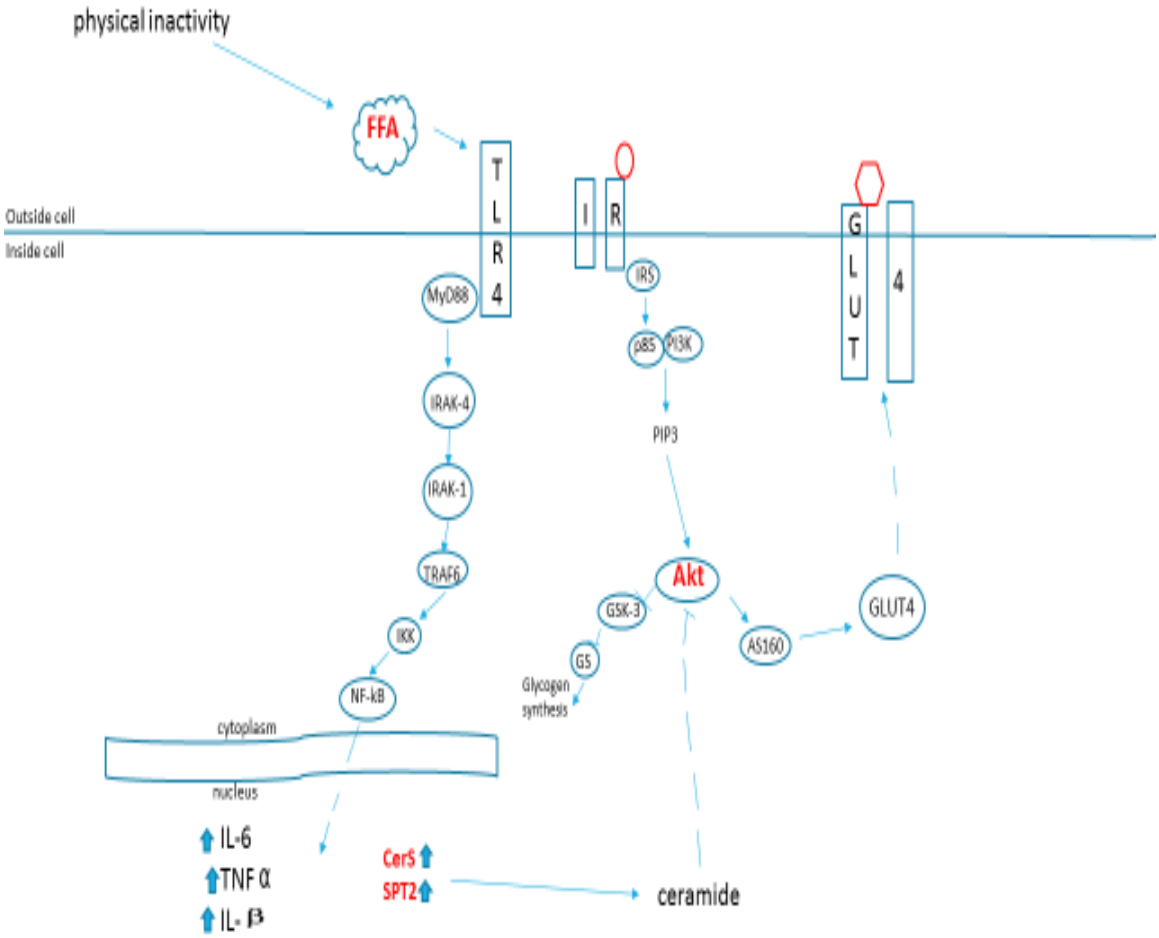


Figure 1: TLR4 and insulin cell signaling in human skeletal muscle

Objective #1: Determine if Akt signaling and ceramide biosynthesis gene expression of SPT1, SPT2, CerS1, CerS2 is altered after 5 days of bed rest in older adults.

Objective #2: Compare insulin, glucose and NEFA before and after 5 days of bed rest in older adults.

METHODS

Screening of participants

We recruited 9 healthy male and female older adult participants (age range: 60-75). All subjects were recruited through advertisements in the Salt Lake City community. The subjects were recreationally active, but were not engaged in any regular exercise training program currently, as defined by two or more exercise training sessions of moderate to high intensity aerobic or resistance exercise per week. Exclusion criteria include, but are not limited to: heart, lung, blood, vascular, liver, kidney, infectious, oncologic, and neurological diseases. All subjects gave their written informed consent before participating in the study, which was approved by the Institutional Review Board of the University of Utah.

Experimental design/bed rest

Research participants underwent a 5-day bed rest experiment in the Center for Clinical and Translational Science at the University of Utah Hospital. Participants arrived at the CCTS at 0600 on day 1 (with 48h refrainment of intense physical activity) and remained in their hospital bed except for bathroom and hygiene privileges until the afternoon of day 5 (~1500). Over the bed rest period, non-pharmacological deep venous thrombosis prevention was performed which consisted of intermittent lower leg compression devices, compression stockings, and daily passive range of motion by a physical therapist. Bathing and hygiene activities were performed at the sink while sitting in a wheel chair. Toilet privileges were provided with the use of a wheel chair and the transportation assistance of the nurses. Adherence to bed rest was continuously monitored and reinforced daily by nursing staff and study personnel. Safety blood samples (PT, PTT, d-dimer) were taken in the morning of each day of bed rest under fasting conditions. These

samples were also used to measure fasting levels of insulin, glucose and fatty acid concentrations (see Figure 2 for bed rest timeline).

Muscle biopsy procedure

After an overnight fast, percutaneous muscle biopsies were collected in the morning of the initiation of bed rest day 1 (pre bed rest) and at the same time in the morning of bed rest day 5 (post bed rest). Muscle biopsies were taken from the vastus lateralis using aseptic technique, local anesthesia (1% lidocaine), and a 5mm Bergström biopsy needle with manual suction. All muscle tissue were immediately blotted and dissected of visible non-muscle tissue, flash-frozen in liquid nitrogen and stored at -80˚C.

Oral glucose tolerance test

On an alternate day before the bed rest study, participants took part in an oral glucose tolerance test. Subjects were fasted (~8h). Plasma glucose and insulin concentrations were measured at baseline (immediately before the glucose drink) and at every 15 min for the first hour and then every 30 min for the following 2h. The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated from the fasting plasma glucose and insulin concentrations (31). The Matsuda index was calculated from plasma and glucose levels during the OGTT (32). The oral glucose tolerance test was repeated on day 4 of bed rest to determine the effects of bed rest on insulin sensitivity. The HOMA-IR was also calculated on day 5 using the fasting blood sample.

Blood insulin and fatty acids concentrations

Fasting blood samples collected pre bed rest (day 1) and on the morning of day 4-5 were used to measure plasma insulin (Millipore) and glucose (YSI). Fasting blood samples collected on day 1 and day 5 were also used to measure plasma NEFAs using the NEFA C enzymatic assay kit (WAKO, Neuss, Germany). Plasma LPS concentration was measured by a

commercially available kit (Cambrex Limulus Amebocyte Lysate [LAL] kit; Lonza, Walkersville, MD).

Western blot

Approximately 30mg of frozen muscle was homogenized (1:9, w/v) in a buffer containing: 50 mM Tris-HCl, 250 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, pH 7.4, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF and 5 μg ml−1 soybean trypsin inhibitor. Muscle homogenates were centrifuged, supernatant collected, and protein concentration determined using a spectrophotometer (EPOCH; BioTek, Winooski, VA). Sample buffer (2X) was added (1:1) to an aliquot of supernatant then the samples were boiled for 3 min. An aliquot of protein (40ug) was loaded on a polyacrylamide gel (BioRad, Hercules, CA) then separated with SDS-PAGE for 1h at 150V. Protein was transferred (50V, 1h) to a polyvinylidene diflouride membrane then blocked for 45 min at room temperature with 2% NFDM in Tris-buffered saline in 0.1% Tween-20 (TBST) on a rocker. Membranes were incubated overnight in a primary antibody solution diluted 1:1000 in 2% NFDM with p-Akt (Ser473 and Thr308) antibody from Cell Signaling Technologies (catalog #9271 and #9275). The next morning, blots were rocked in secondary antibody (1:6000; Santa Cruz Biotechnology, Santa Cruz, CA) for 1h at room temperature then washed 3x (5 min) with TBST. Chemiluminescence reagent (ECL Plus, GE Healthcare) was applied to each blot then incubated for 5 min at room temperature. Optical density measurement was obtained with a digital imager (ChemiDoc XRS+, BioRad). Membranes that contain phosphorylated proteins were stripped (Restore Western Blot Stripping Buffer; Pierce Biotechnlogy, CA) of primary and secondary antibodies then re-probed for total Akt (1:1000, catalog #9272, Cell Signaling). Densitometric analysis was performed using Lab version 4.1 software (BioRad). Band density was normalized to an internal control sample (loaded in duplicate on each gel). Replicate samples were averaged and reported as fold change from basal. Results were reported relative to their total protein content (p-Akt/total Akt).

Statistical analysis

Analysis of protein expression in older adult skeletal muscle was conducted using a paired t test. An area under the curve was determined for glucose and insulin values collected from the OGTT and were compared before and after bed rest. Insulin and glucose area under the curve obtained from the OGTT and baseline insulin, glucose and NEFA concentrations was analyzed using a 2-way ANOVA (Age, Time). Pearson correlations were used on select muscle markers and physiological parameters. All values were presented as mean ± SE. Significance was set at $p \leq 0.05$. All analyses were performed with SigmaPlot (Version 12.0).

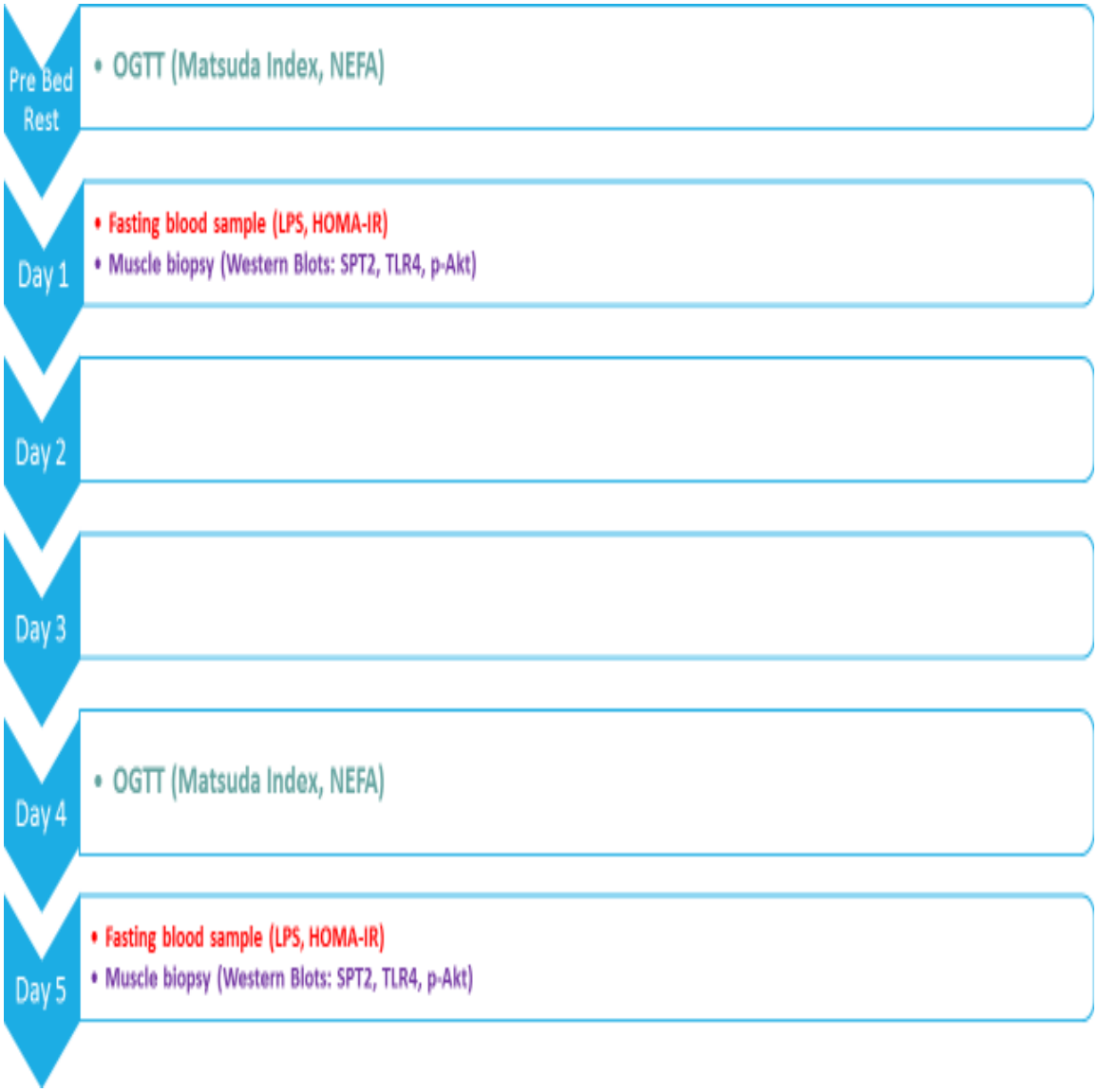


Figure 2: Bed rest timeline

RESULTS

The HOMA-IR was calculated from the fasting plasma glucose and insulin concentrations. There was no difference in HOMA-IR (**Figure 3**) post bed rest (p=0.50). The Matsuda index was calculated from plasma and glucose levels during the OGTT. Matsuda Index (**Figure 4**) decreased after bed rest. (p=0.02).

Glucose levels increased at 60 and 90 min post bed rest during the OGTT when compared to pre bed rest (**Figure 5A,** p<0.05). Total OGTT glucose AUC was higher for post bed rest versus pre bed rest (**Figure 5B**, p<0.05). Serum insulin levels during the OGTT were elevated after bed rest at all the time points (**Figure 6A**, P<0.05). Total insulin AUC tended to be higher after bed rest (**Figure 6B**, P=0.06).

Fasting NEFA levels were not different after bed rest (**Figure 7A**, p=0.84). Nor were there any changes in NEFA levels during an OGTT after bed rest (**Figure 7B**, (Total NEFA AUC: p=0.36). Following bed rest, fasting LPS levels did not change but there was a tendency for LPS levels to increase when normalized to pre bed rest levels (91%; **Figure 8**, p=0.09).

Following bed rest, TLR4 protein expression tended to increase by ~35% (**Figure 9A**, P=0.08), SPT2 increased by ~40% (**Figure 9B**, P<0.05) and Akt phosphorylation of Ser473 decreased by ~20% (**Figure 9C**, P<0.05). No difference was seen in the Akt phosphorylation of Thr308 (**Figure 9D**).

Finally, we determined the relationship of two of our notable skeletal muscle findings with glucose uptake impairment in older adults after bed rest. We found that after 5 days of bed rest, TLR4 protein expression was inversely correlated with the Matsuda index (**Figure 10A**) and positively correlated with insulin AUC (**Figure 10B**) (P<0.05) but not glucose AUC (**Figure 10C**). In contrast, SPT2 protein expression was not related to either of these physiological parameters

(**Figures 10D-F**). Additionally, HOMA-IR was not correlated with TLR4 (R=0.23) or SPT2 (R=0.40) protein content (not shown).

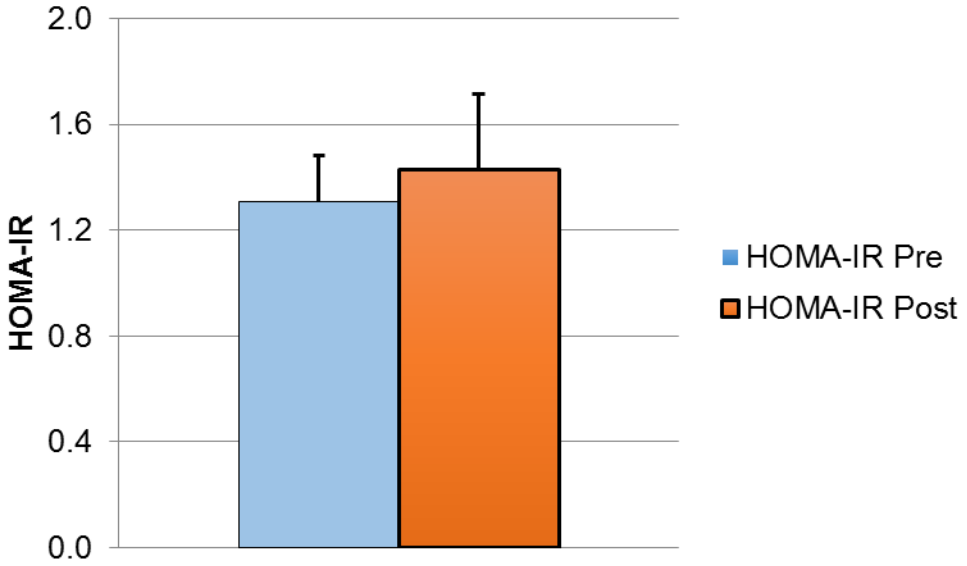


Figure 3: HOMA-IR pre bed rest value 1.3 ± 0.2, post bed rest value 1.4 ± 0.3 (p=0.50). Values are Mean±SE.

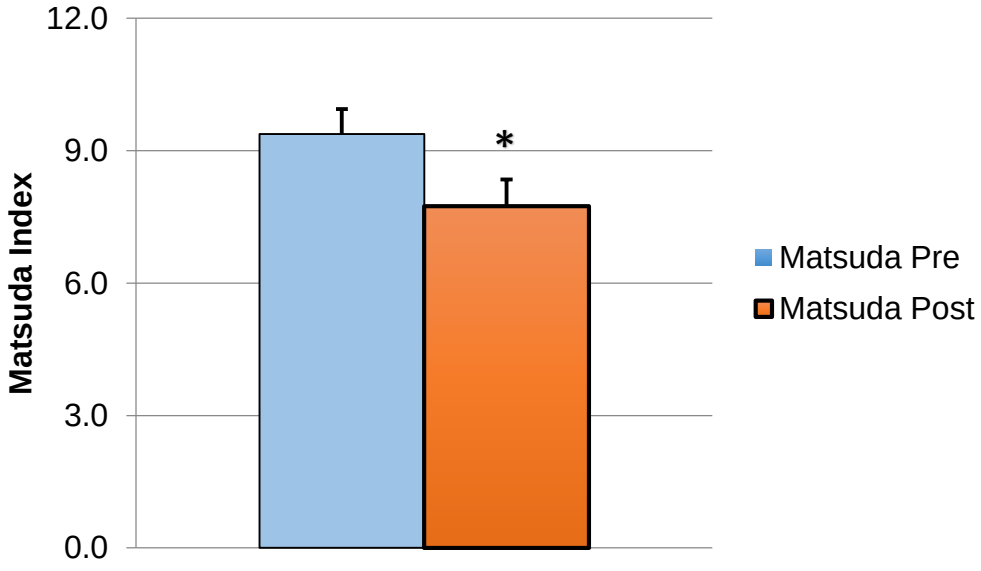
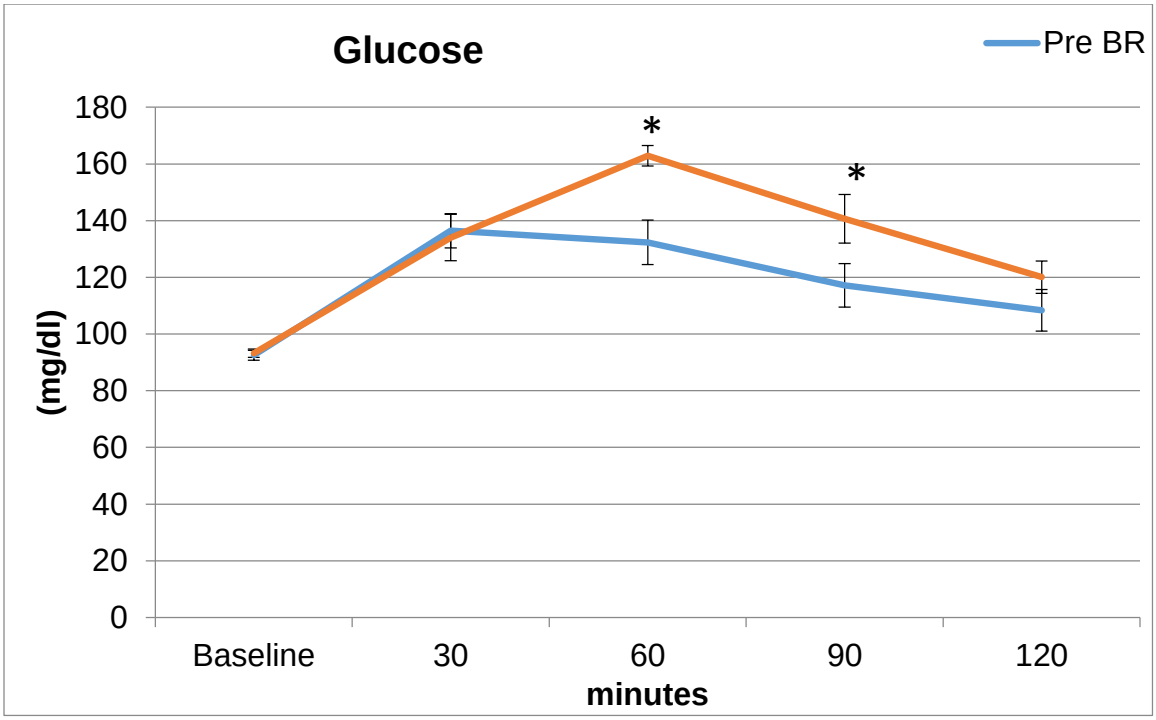


Figure 4: Matsuda index pre bed rest value 9.4 ± 0.6, post bed rest value 7.7 ± 0.6 (p=0.02). Values are Mean±SE.

A



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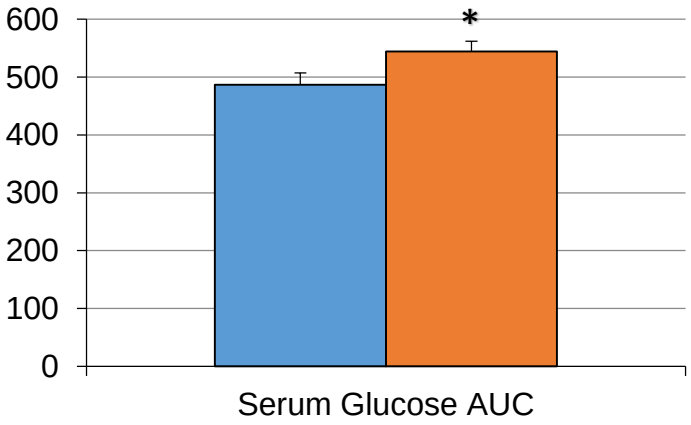
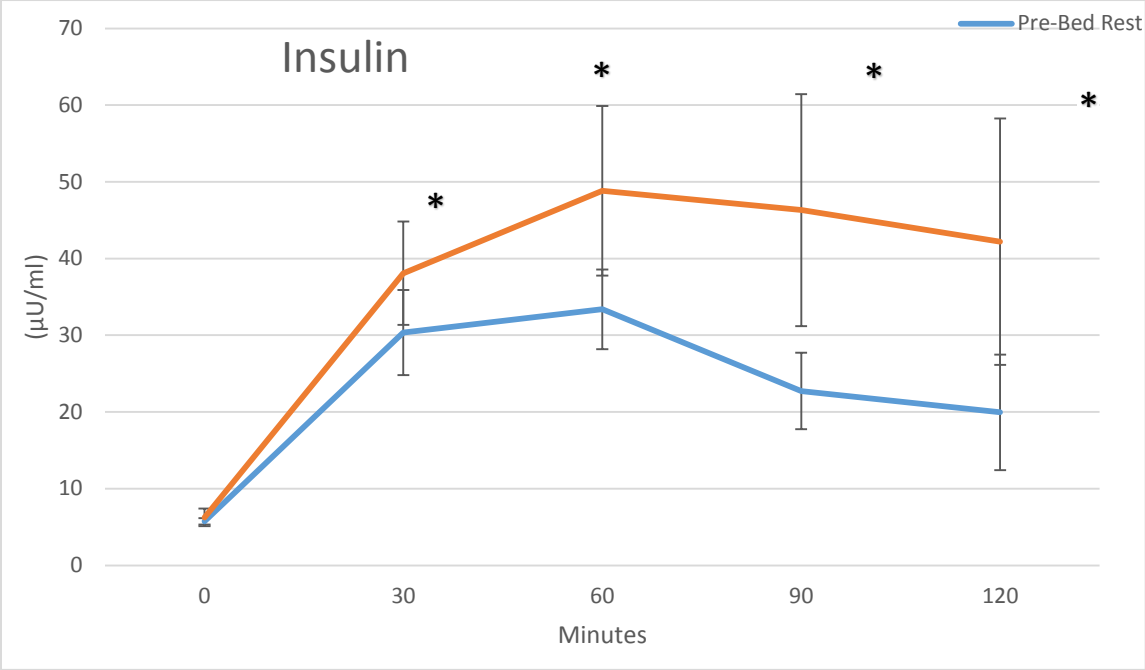


Figure 5: Glucose. **A:** Glucose levels higher at 60 and 90 min during the OGTT after bed rest compared to pre bed rest (* indicates $p<0.05$). **B:** Total glucose AUC was 486.4 for pre bed rest versus 544.2 for post bed rest ($p<0.05$). Values are Mean±SE.

A



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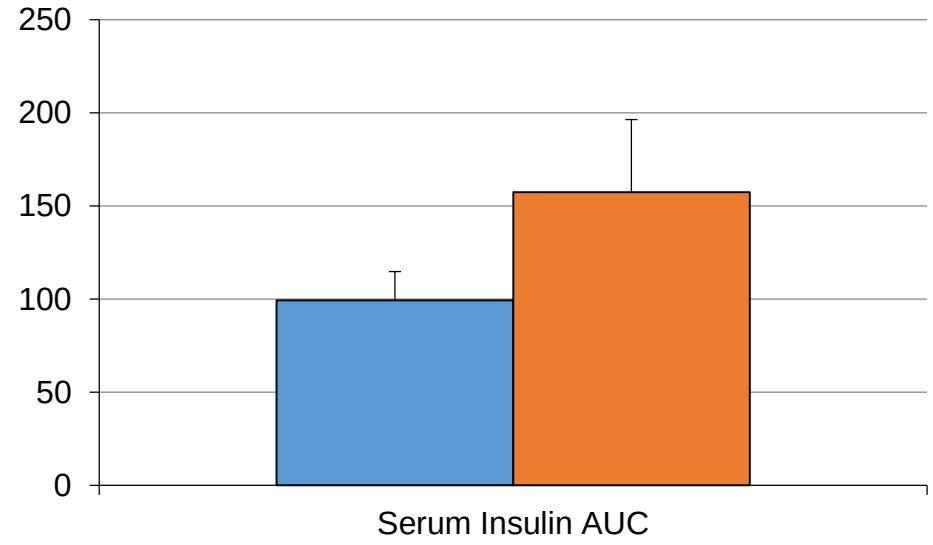
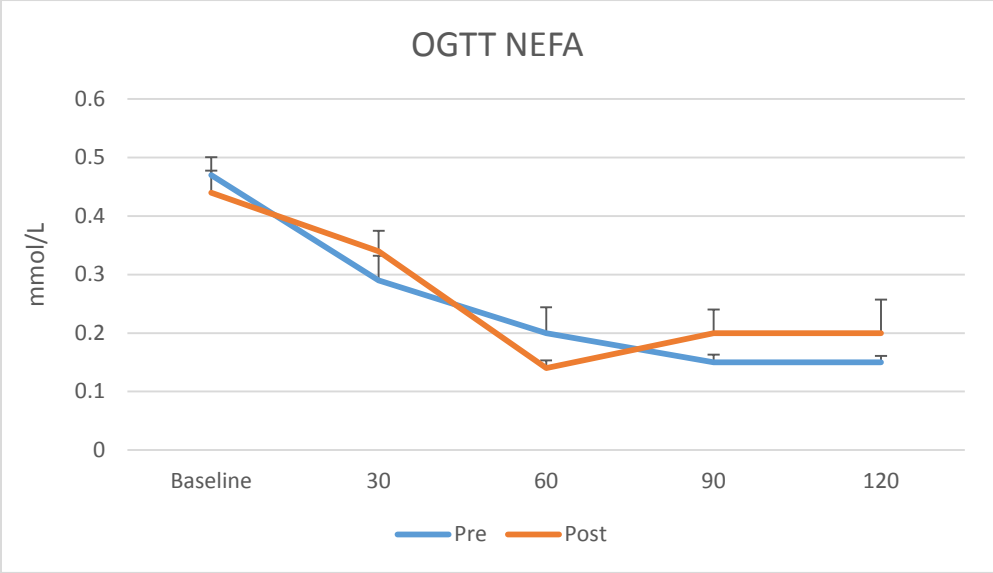


Figure 6: Insulin. **A:** Serum insulin levels during OGTT before and after bed rest (* indicates P<0.05). **B:** Total insulin AUC (P=0.06). Values are Mean±SE.

A



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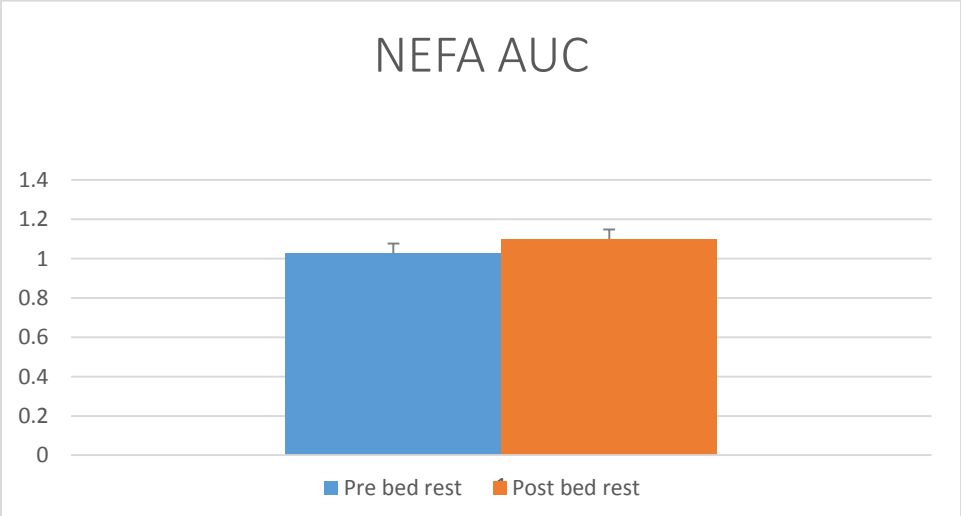
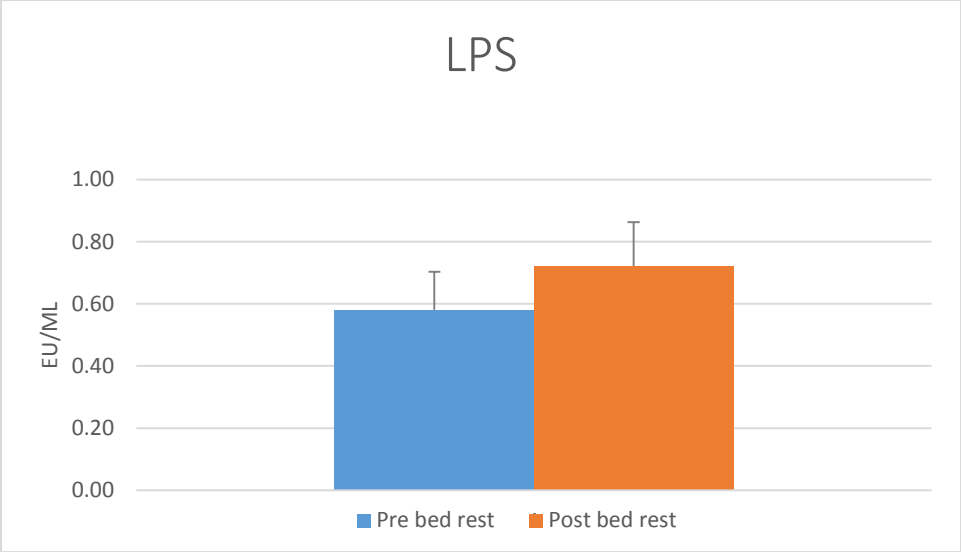


Figure 7: NEFA. **A:** Non Esterified Fatty Acids during OGTT (p=0.84). **B:** Total AUC pre bed rest 1.03 ± 0.05 and post bed rest 1.10 ± 0.05 (p=0.83). Values are Mean±SE.

A



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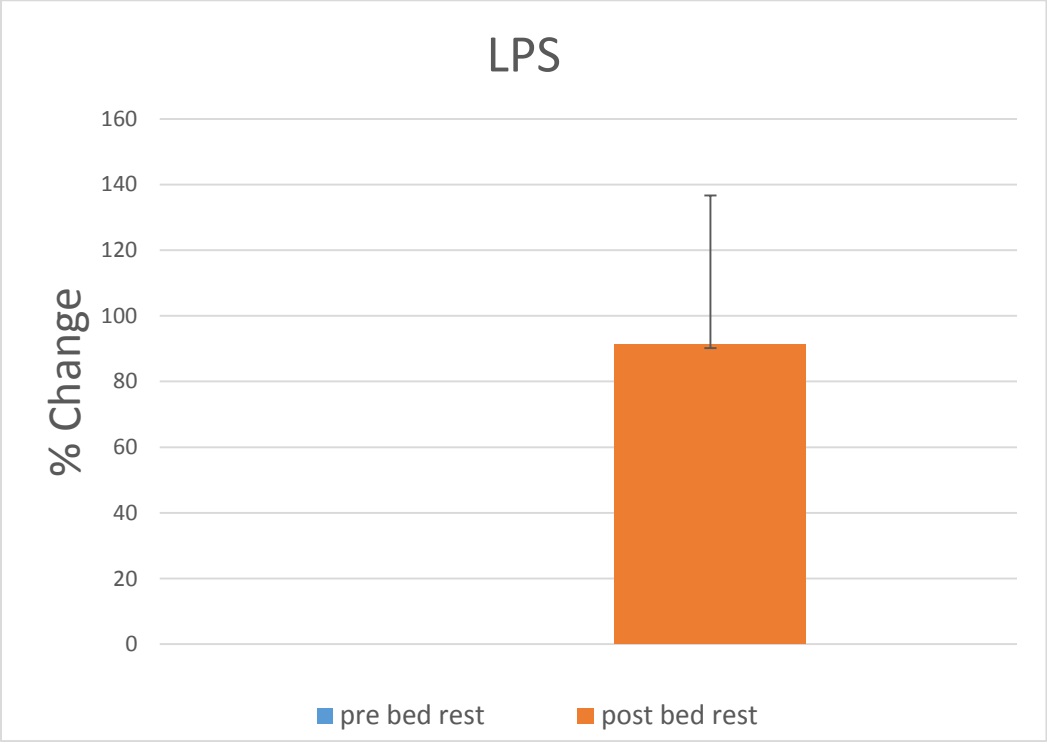

Figure 8: LPS. **A:** Lipopolysaccharides pre 0.58 ± 0.12 and post bed rest 0.72 ± 0.14 (p=0.46). **B:** Lipopolysaccharides % Chg: 91.2 ± 45.5 (p=0.09). Values are Mean±SE.

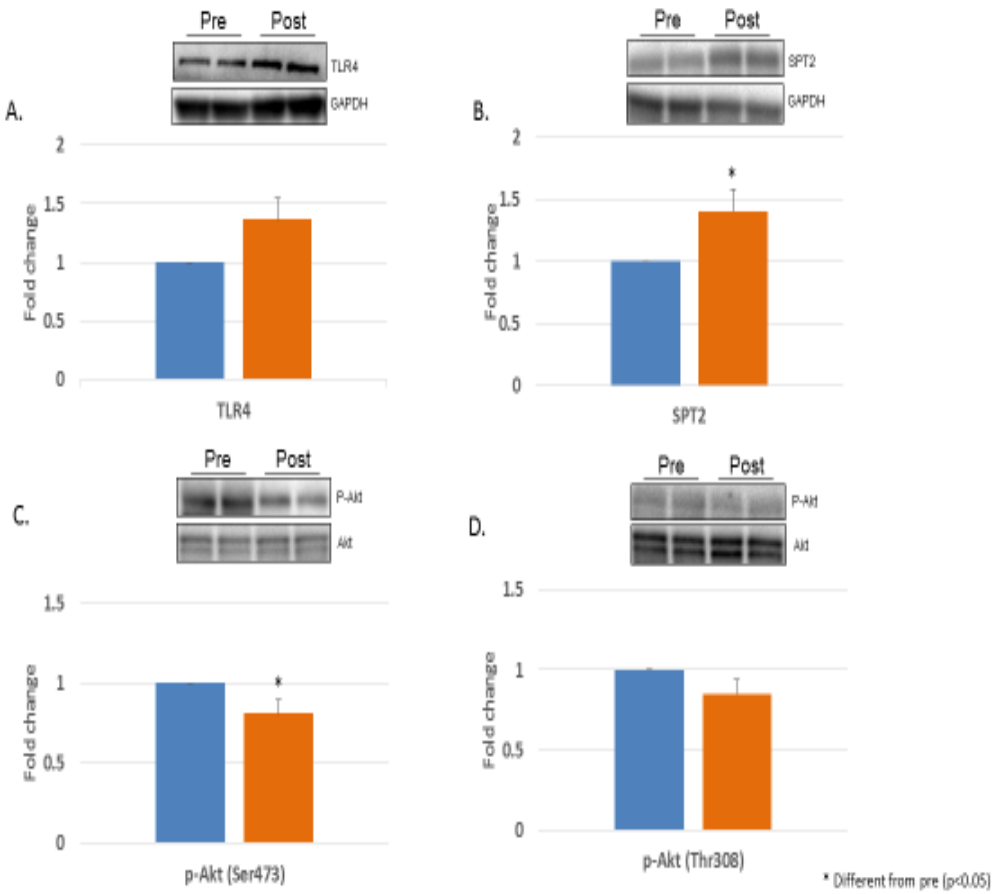

Figure 9: Western blots. **A:** TLR4 protein expression (P=0.08). **B:** SPT2 protein expression (P<0.05). **C:** Akt phosphorylation at Ser473 (P<0.05). **D:** Akt phosphorylation at Thr308 (NS). Data were determined at fasting levels before (Pre) and after (Post) bed rest. Values are Mean±SE. Insets above figures are examples of western blot images.

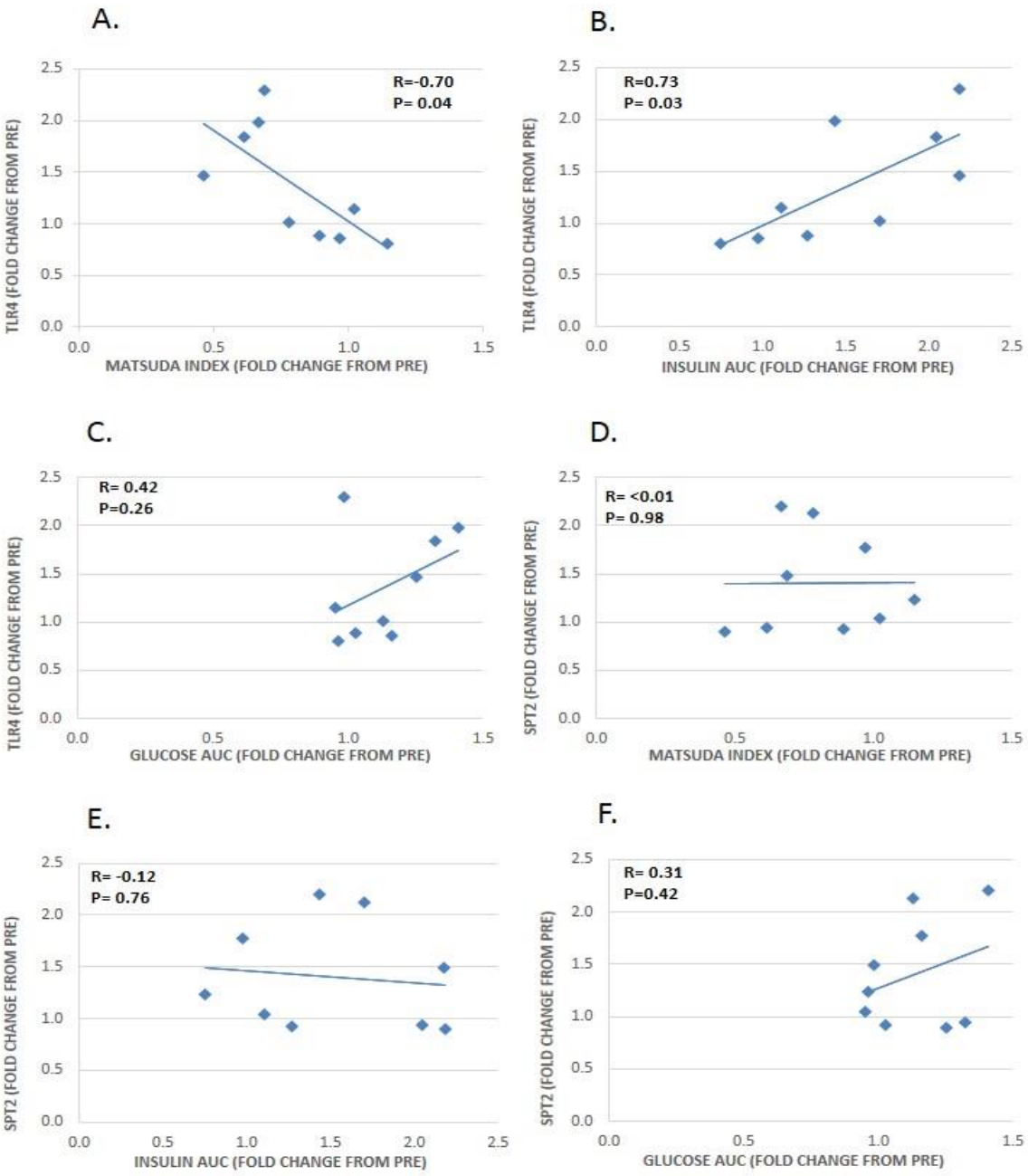

Figure 10: Correlations. **A:** Correlation between TLR4 and Matsuda index (p=0.04). **B:** Correlation between TLR4 and insulin AUC (p=0.03). **C:** Correlation between TLR and glucose AUC (NS). **D:** Correlation between SPT2 and Matsuda index (NS). **E:** Correlation between SPT2 and insulin AUC (NS). **F:** Correlation between SPT2 and glucose AUC (NS).

DISCUSSION

In the current study, our primary finding was that 5 days of bed rest impaired skeletal muscle insulin signaling (Akt phosphorylation) and increased ceramide biosynthesis signaling (STP2) protein expression in older adults. Interestingly, increased TLR4 protein content after bed rest was strongly correlated to postprandial glucose and insulin responses. These data support the hypothesis that short-term physical inactivity (i.e., controlled bed rest) alters skeletal muscle ceramide biosynthesis expression**,** and may be related to skeletal muscle insulin sensitivity and whole body glucose disposal in older adults.

Nutritional overload and physical inactivity are two major contributors to metabolic disruption. Unfortunately, less is known of the metabolic consequences of a short duration of physical inactivity in older adults. We show for the first time that a brief period of bed rest in older adults can increase SPT2 by ~40%, a critical enzyme responsible for ceramide synthesis. This finding is consistent with a recent study by Holland and colleagues that reported an upregulation of SPT2 mRNA following a lipid infusion into skeletal muscle of mice (5). In another recent study by Hansen et al., skeletal muscle cells of mice that were treated with insulin and palmitate elicited an accumulation in ceramides due to an increase in expression of SPT2, and when SPT2 was knocked out, this prevented an increase in ceramides (33). Although ceramide was not measured in the current study, the importance of SPT2 in regulating de novo ceramide levels can be seen in studies that use myriocin, a potent inhibitor of SPT2. When used, myriocin can prevent lipid-induced insulin resistance and ceramide accumulation in skeletal muscle of rodents (5), (34). Ceramide has been tied to insulin resistance and other metabolic abnormalities such as atherosclerosis, cardiomyopathy, and diabetes. One mechanism by which ceramides are believed to impair insulin signaling is by inhibiting Akt phosphorylation. For example, in a study by Powell et al., incubation of muscle cells with palmitate promoted intracellular accumulation of

ceramide and inhibited Akt signaling by activating PKCζ, which negatively regulates Akt by suppressing its cell-surface recruitment and phosphorylation (30). However, when muscle cells were incubated with myriocin, this suppressed ceramide synthesis and antagonized the inhibitory effect of palmitate on Akt activation and regained insulin stimulated glucose transport (30). Therefore, it is plausible that an increase in ceramide production may have participated in the ~20% reduction in Akt phosphorylation seen in this study following bed rest in older adults. However, future studies are needed to evaluate if increased enzyme activity with bed rest results in ceramide species accumulation in muscle tissue before these conclusions can be drawn.

We cannot decipher the cause of increased SPT2 protein expression after bed rest in skeletal muscle tissue of older adults. However, it is possible that the TLR4 signaling pathway may be partly involved. Holland et al. determined that TLR4 was necessary for de novo ceramide synthesis and upregulation of ceramide biosynthesis genes such as SPT2 as determined by experiments that injected LPS into skeletal muscle of mice and separate experiments in TLR4 KO animals (5). In our study, we showed a tendency for skeletal muscle TLR4 protein abundance and a circulating TLR4 ligand (LPS) to increase after bed rest. Additionally, there was a strong correlation between an increase in TLR4 and altered post prandial glucose metabolism in these older adults after bed rest. Possibly a longer period of bed rest may be required to significantly increase TLR4 above pre bed rest levels as seen in a similar, albeit longer, 7-day bed rest study in older adults (25). Alternately, it is possible that a bed rest-induced increase in SPT2 could operate through a different TLR protein, since many TLRs and even some IL-receptors signal through NFκB. For example, a previous study in humans showed a transcriptional increase in not only skeletal muscle TLR4 but also TLR5 and 6 in response to a 2-day lipid infusion (27). In another study, inhibition of TLR2 in cultured muscle cells of mice resulted in a near complete inhibition of palmitate-induced insulin resistance (35). Finally, activation of the TLR4 signaling pathway may occur independent of an increase in TLR4 protein or ligand abundance since many of the downstream TLR4 adaptor and signaling molecules (e.g., MyD88, TIRAP, IRAKs, TRAFs) are regulated by protein-protein interactions and dimerization steps. Future studies are needed

to evaluate a larger range of TLR proteins and evaluation of downstream signaling proteins within the TLR4 pathway with 5 days of bed rest in skeletal muscle of older adults.

Although a previous study in older adults showed that 10 days of bed rest altered glucose handling our study supports that as little as 5 days was sufficient to disrupt post-prandial glucose disposal in older adults (as determined by an OGTT and the Matsuda index). This finding may be intricately tied to the impaired Akt signaling and increased SPT2 protein abundance we observed in skeletal muscle homogenate samples of older adults especially since skeletal muscle is the primary source of post-prandial glucose disposal. On the contrary, we did not note any difference in the HOMA-IR and post-prandial NEFA disposal as opposed to 10 days of bed rest in older adults by Coker et al. (3) and also in an age-related study by Ghosh et al. [36]. These differences suggest that 5 days was sufficient to cause skeletal muscle dysfunction but additional days of bed rest (e.g., 10d) may affect metabolism of other organs (liver) and alter post-prandial fuel utilization. Nonetheless, our data are important and clinically relevant because: 1) they clearly demonstrate the metabolic potency of very short-term bouts of inactivity in older adults; 2) a majority of older adults hospitalized for acute illness typically spend ~5 days with low amounts of ambulatory activity during hospitalization (15). Therefore, future studies are needed to evaluate inpatients interventions such as early mobility and the minimal amount of contractile activity to combat early skeletal muscle metabolic decline in older adults during periods of inactivity such as acute hospitalization/bed rest.

In conclusion, we show for the first time that 5 days of bed rest in older adults can increase SPT2 protein expression, a major regulator of ceramide biosynthesis signaling and impair skeletal muscle insulin signaling via Akt phosphorylation. TLR4-mediated SPT2 expression after bed rest may be an important link between physical inactivity and glucose intolerance as evidenced by a positive correlation between increased TLR4 protein content and postprandial glucose and insulin responses. Taken together, these data will be important for development of future pharmacological interventions aimed at reducing skeletal muscle TLR4 and SPT2 signaling and ultimately maintenance of post-prandial glucose tolerance in physically inactive older adults.

APPENDIX

SUBJECT CHARACTERISTICS AND PHYSIOLOGICAL PARAMETERS BEFORE AND AFTER BED REST

Subject characteristics and physiological parameters before and after bed rest									
		Pre Bed Rest				Post Bed Rest			p-value
Age, yrs		66	±	1			-		-
BMI, kg/m2		25	±	1			-		-
Body Weight, kg		71.6	±	3.9		70	±	4	<0.01
Total Lean Mass, %		60.3	±	2		59.1	±	2	<0.01
Total Fat Mass, %		36.6	±	2		37.7	±	2	<0.01
Fasting Glucose, mg/dL		92.5	±	1.8		93.2	±	1.5	0.64
Fasting Insulin, µU/mL		5.7	±	0.4		6.2	±	0.6	0.54
NEFA, mmoL/L		0.42	±	0.04		0.4	±	0.09	0.84
NEFA, % Chg		-		-		1.1	±	0.17	0.71
High Density Lipoprotein (HDL), mg/dL		55.8	±	3.1		49.8	±	2.5	0.02
Low Density Lipoprotein (LDL), mg/dL		92.7	±	7.9		97.9	±	10.1	0.38
Very Low Density Lipoprotein, mg/dL		18.7	±	1.4		21.8	±	4.7	0.43
LDL/HDL ratio		1.7	±	0.2		2	±	0.2	0.04
Total Cholesterol, mg/dL		167.1	±	8.8		169.2	±	10.3	0.71
Triglycerides, mg/dL		93.4	±	7.1		109.8	±	23.7	0.4
	Data are from from n=9 older adults and reported as Mean±SE.								

REFERENCES

1. Waxman A. Why a global strategy on diet, physical activity and health? Wld Rev Nutr Diet 2005;95(R):162.

2. Breen L, Stokes KA, Churchward-Venne TA, Moore DR, Baker SK, Smith K, Atherton PJ, Phillips SM. Two weeks of reduced activity decreases leg lean mass and induces "anabolic resistance" of myofibrillar protein synthesis in healthy elderly. J Clin Endocrinol Metab 2013;98(6):2604-12. doi: 10.1210/jc.2013-1502.

3. Coker RH, Hays NP, Williams RH, Xu L, Wolfe RR, Evans WJ. Bed rest worsens impairments in fat and glucose metabolism in older, overweight adults. J Gerontol A Biol Sci Med Sci 2014;69(3):363-70. doi: 10.1093/gerona/glt100.

4. Reaven GM. The insulin resistance syndrome: definition and dietary approaches to treatment. Annu Rev Nutr 2005;25:391-406. doi: 10.1146/annurev.nutr.24.012003.132155.

5. Holland WL, Bikman BT, Wang LP, Yuguang G, Sargent KM, Bulchand S, Knotts TA, Shui G, Clegg DJ, Wenk MR, et al. Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice. J Clin Invest 2011;121(5):1858-70. doi: 10.1172/JCI43378.

6. Bienso RS, Ringholm S, Kiilerich K, Aachmann-Andersen NJ, Krogh-Madsen R, Guerra B, Plomgaard P, van Hall G, Treebak JT, Saltin B, et al. GLUT4 and glycogen synthase are key players in bed rest-induced insulin resistance. Diabet 2012;61(5):1090-9. doi: 10.2337/db11-0884.

7. Amati F, Dube JJ, Coen PM, Stefanovic-Racic M, Toledo FG, Goodpaster BH. Physical inactivity and obesity underlie the insulin resistance of aging. Diabet Care 2009;32(8):1547-9. doi: 10.2337/dc09-0267.

8. Drummond MJ, Addison O, Brunker L, Hopkins PN, McClain DA, LaStayo PC, Marcus RL. Downregulation of E3 ubiquitin ligases and mitophagy-related genes in skeletal muscle of physically inactive, frail older women: a cross-sectional comparison. J Gerontol A Biol Sci Med Sci 2014;69(8):1040-8. doi: 10.1093/gerona/glu004.

9. Stuart CA, Shangraw RE, Prince MJ, Peters EJ, Wolfe RR. Bed-rest-induced insulin resistance occurs primarily in muscle. Metab 1988;37(8):802-6.

10. Mikines KJ, Richter EA, Dela F, Galbo H. Seven days of bed rest decrease insulin action on glucose uptake in leg and whole body. J Appl Phys 1991;70(3):1245-54.

11. Blanc S, Normand S, Pachiaudi C, Fortrat J-O, Laville M, Gharib C. Fuel homeostasis during physical inactivity induced by bed rest 1. J Clin Endocrinol Metab 2000;85(6):2223-33.

12. Yanagibori R, Suzuki Y, Kawakubo K, Makita Y, Gunji A. Carbohydrate and lipid metabolism after 20 days of bed rest. Acta Phys Scand Supp 1993;616:51-7.

13. Duran-Valdez E, de Serna DG, Schneider S, Amorim F, Burge M, Schade DS. Metabolic effects of 2 days of strict bed rest. Endocr Pract 2008;14(5):564-9.

14. Smorawinski J, Kaciuba-Uscilko H, Nazar K, Kubala P, Kaminska E, Ziemba A, Adrian J, Greenleaf J. Effects of three-day bed rest on metabolic, hormonal and circulatory responses to an oral glucose load in endurance or strength trained athletes and untrained subjects. J Phys Pharmacol 2000;51(2).

15. Fisher SR, Kuo Y-f, Graham JE, Ottenbacher KJ, Ostir GV. Early ambulation and length of stay in older adults hospitalized for acute illness. Archi Int Med 2010;170(21):1942-3.

16. Knudsen SH, Hansen LS, Pedersen M, Dejgaard T, Hansen J, Hall GV, Thomsen C, Solomon TP, Pedersen BK, Krogh-Madsen R. Changes in insulin sensitivity precede changes in body composition during 14 days of step reduction combined with overfeeding in healthy young men. J Appl Physiol (1985) 2012;113(1):7-15. doi: 10.1152/japplphysiol.00189.2011.

17. Zierath J, He L, Guma A, Wahlström EO, Klip A, Wallberg-Henriksson H. Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. Diabet 1996;39(10):1180-9.

18. Keller SR, Lienhard GE. Insulin signalling: the role of insulin receptor substrate 1. Tren Cell Bio 1994;4(4):115-9.

19. Calleja V, Alcor D, Laguerre M, Park J, Vojnovic B, Hemmings BA, Downward J, Parker PJ, Larijani B. Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo. PLoS Biol 2007;5(4):e95. doi: 10.1371/journal.pbio.0050095.

20. Hanada M, Feng J, Hemmings BA. Structure, regulation and function of PKB/AKT--a major therapeutic target. Biochim Biophys Acta 2004;1697(1-2):3-16. doi: 10.1016/j.bbapap.2003.11.009.

21. Miinea CP, Sano H, Kane S, Sano E, Fukuda M, Peranen J, Lane WS, Lienhard GE. AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. Biochem J 2005;391(Pt 1):87-93. doi: 10.1042/BJ20050887.

22. Højlund K, Stæhr P, Hansen BF, Green KA, Hardie DG, Richter EA, Beck-Nielsen H, Wojtaszewski JF. Increased phosphorylation of skeletal muscle glycogen synthase at NH2-terminal sites during physiological hyperinsulinemia in type 2 diabetes. Diabet 2003;52(6):1393-402.

23. Hayden MS, Ghosh S. NF-kappaB in immunobiology. Cell Res 2011;21(2):223-44. doi: 10.1038/cr.2011.13.

24. Li H, Malhotra S, Kumar A. Nuclear factor-kappa B signaling in skeletal muscle atrophy. J Mol Med (Berl) 2008;86(10):1113-26. doi: 10.1007/s00109-008-0373-8.

25. Drummond MJ, Timmerman KL, Markofski MM, Walker DK, Dickinson JM, Jamaluddin M, Brasier AR, Rasmussen BB, Volpi E. Short-term bed rest increases TLR4 and IL-6 expression in skeletal muscle of older adults. Am J Physiol Regul Integr Comp Physiol 2013;305(3):R216-23. doi: 10.1152/ajpregu.00072.2013.

26. Miles JM, Wooldridge D, Grellner WJ, Windsor S, Isley WL, Klein S, Harris WS. Nocturnal and postprandial free fatty acid kinetics in normal and type 2 diabetic subjects effects of insulin sensitization therapy. Diabet 2003;52(3):675-81.

27. Hussey SE, Lum H, Alvarez A, Cipriani Y, Garduno-Garcia J, Anaya L, Dube J, Musi N. A sustained increase in plasma NEFA upregulates the Toll-like receptor network in human muscle. Diabet 2014;57(3):582-91. doi: 10.1007/s00125-013-3111-x.

28. Summers SA. Ceramides in insulin resistance and lipotoxicity. Prog Lipid Res 2006;45(1):42-72. doi: 10.1016/j.plipres.2005.11.002.

29. Hsieh CT, Chuang JH, Yang WC, Yin Y, Lin Y. Ceramide inhibits insulin-stimulated Akt phosphorylation through activation of Rheb/mTORC1/S6K signaling in skeletal muscle. Cell Sig 2014;26(7):1400-8. doi: 10.1016/j.cellsig.2014.03.004.

30. Powell D, Turban S, Gray A, Hajduch E, Hundal H. Intracellular ceramide synthesis and protein kinase Czeta activation play an essential role in palmitate-induced insulin resistance in rat L6 skeletal muscle cells. Biochem J 2004;382:619-29.

31. Matthews D, Hosker J, Rudenski A, Naylor B, Treacher D, Turner R. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabet 1985;28(7):412-9.

32. Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabet Care 1999;22(9):1462-70.

33. Hansen ME, Tippetts TS, Anderson MC, Holub ZE, Moulton ER, Swensen AC, Prince JT, Bikman BT. Insulin increases ceramide synthesis in skeletal muscle. J Diabetes Res 2014;2014:765784. doi: 10.1155/2014/765784.

34. Watson ML, Coghlan M, Hundal HS. Modulating serine palmitoyl transferase (SPT) expression and activity unveils a crucial role in lipid-induced insulin resistance in rat skeletal muscle cells. Biochem J 2009;417(3):791-801. doi: 10.1042/BJ20081149.

35. Senn JJ. Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. J Biol Chem 2006;281(37):26865-75. doi: 10.1074/jbc.M513304200.

36. Ghosh S, Lertwattanarak R, Garduño JJ, Galeana JJ, Li J, Zamarripa F, Lancaster JL, Mohan S, Hussey S, Musi N: Elevated muscle TLR4 expression and metabolic endotoxemia in human aging. J Gerontol A Biol Sci Med Sci 2015;70(2):232-46. doi: 10.1093/gerona/glu067.